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Regulation of Mucosal Addressin Cell Adhesion Molecule 1 Expression in Human and Mice by Vascular Adhesion Protein 1 Amine Oxidase Activity

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Primary sclerosing cholangitis (PSC) and autoimmune hepatitis are hepatic complications associated with inflammatory bowel disease (IBD). The expression of mucosal addressin cell adhesion molecule 1 (MAdCAM-1) on mucosal endothelium is a prerequisite for the development of IBD, and it is also detected on the hepatic vessels of patients with liver diseases associated with IBD. This aberrant hepatic expression of MAdCAM-1 results in the recruitment of effector cells initially activated in the gut to the liver, in which they drive liver injury. However, the factors responsible for the aberrant hepatic expression of MAdCAM-1 are not known. In this study, we show that deamination of methylamine (MA) by vascular adhesion protein 1 (VAP-1) [a semicarbazide-sensitive amine oxidase (SSAO) expressed in the human liver] in the presence of tumor necrosis factor α induces the expression of functional MAdCAM-1 in hepatic endothelial cells and in intact human liver tissue *ex vivo*. This is associated with increased adhesion of lymphocytes from patients with PSC to hepatic vessels. Feeding mice MA, a constituent of food and cigarette smoke found in portal blood, led to VAP-1/SSAO–dependent MAdCAM-1 expression in mucosal vessels *in vivo*. *Conclusion:* Activation of VAP-1/SSAO enzymatic activity by MA, a constituent of food and cigarette smoke, induces the expression of MAdCAM-1 in hepatic vessels and results in the enhanced recruitment of mucosal effector lymphocytes to the liver. This could be an important mechanism underlying the hepatic complications of IBD. (HEPATOLOGY **2011;53:661-672**)

Mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is a 60-kDa endothelial cell adhesion molecule that is constitutively expressed on high endothelial venules (HEVs) in Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) and in vessels of the lamina propria.[1-3]

Abbreviations: AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; HCHO, formaldehyde; HEC, hepatic endothelial cell; HEV, high endothelial venule; hVAP-1, human vascular adhesion protein 1; IBD, inflammatory bowel disease; ICAM-1, intercellular cell adhesion molecule 1; IMC, isotype-matched control; MA, methylamine; MAdCAM-1, mucosal addressin cell adhesion molecule 1; MLN, mesenteric lymph node; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; NH_3, ammonia; NL, normal liver; PBC, primary biliary cirrhosis; PBL, peripheral blood lymphocyte; PCR, polymerase chain reaction; PP, Peyer's patch; PSC, primary sclerosing cholangitis; rVAP-1, recombinant vascular adhesion protein 1; sMAdCAM-1, soluble mucosal addressin cell adhesion molecule 1; SSAO, semicarbazide-sensitive amine oxidase; TNF-α, tumor necrosis factor α; VAP-1, vascular adhesion protein 1; VCAM-1, vascular cell adhesion molecule 1; WT, wild type.

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Potential conflict of interest: Dr. Pullen owns stock in Pfizer. Dr. Jalkanen owns stock in Biotec Therapies.

Additional Supporting Information may be found in the online version of this article.

MAdCAM-1 orchestrates the recruitment of lymphocytes into mucosal tissues via interactions with the $\alpha4\beta7$ integrin,[4] and it has been implicated in the sustained destructive gut inflammation that characterizes inflammatory bowel disease (IBD).[3] Its importance has been highlighted by the fact that antibodies directed against either MAdCAM-1 or $\alpha4\beta7$ attenuate inflammation in animal models and patients with colitis[5,6] or Crohn's disease.[7,8]

MAdCAM-1 was initially thought to be a gut-specific molecule[3] but was subsequently found to be induced in the adult human liver in association with portal tract inflammation,[9] in which it could support the adhesion of $\alpha4\beta7^+$ gut-derived lymphocytes.[10] This aberrant hepatic expression of MAdCAM-1 led to the hypothesis that an enterohepatic circulation of long-lived mucosal lymphocytes through the liver could trigger extra-intestinal hepatic inflammation in patients with liver diseases complicating IBD.[11]

Another molecule potentially involved in this enterohepatic lymphocyte recirculation is vascular adhesion protein 1 (VAP-1), an adhesion molecule with amine oxidase activity that supports lymphocyte recruitment to the liver.[12-14] Substrates for VAP-1 include aliphatic amines such as methylamine (MA), which can be detected in portal blood as a result of food consumption.[15] VAP-1 is normally expressed in the human liver and weakly on mucosal vessels; however, it is rapidly induced in inflamed mucosa in patients with IBD.[16] Thus, there is complementarity of expression of VAP-1 and MAdCAM-1 molecules. Moreover, previous reports from our group have shown that deamination of benzylamine by the enzymatic activity of VAP-1 on hepatic endothelium leads to nuclear factor kappa B (NF-κB) activation, increased adhesion molecule expression, and enhanced leukocyte adhesion.[17] Many studies also support the role of tumor necrosis factor α (TNF-α) in inducing MAdCAM-1 expression,[18-20] and because of the colocalization of VAP-1 and MAdCAM-1 on hepatic endothelium and the association of primary sclerosing cholangitis (PSC) with IBD, we hypothesized that TNF-α released from the inflamed gut together with increased levels of MA in portal blood could act via VAP-1/semicarbazide-sensitive amine oxidase (SSAO) activity to induce hepatic MAdCAM-1 expression. Using two *in vitro* human models and *in vivo* studies in mice, we now show that this is the case. We suggest that this is a novel mechanism explaining aberrant hepatic MAdCAM-1 expression in patients with IBD and thus an important pathogenic mechanism in liver diseases complicating IBD.

Materials and Methods

Human Tissue and Blood

Human liver tissue was obtained through the Liver Unit of Queen Elizabeth Hospital. Diseased tissue came from explanted livers removed at transplantation. Nondiseased liver tissue came from either surplus donor tissue (i.e., tissue exceeding transplantation requirements) or surgical resections of liver tissue containing metastatic tumors; in the latter case, uninvolved tissue was taken several centimeters away from any tumor deposits. Whole blood was obtained from patients with PSC and IBD. All human tissue and blood samples were collected with the approval of the local research ethics committee and with patient consent.

Isolation and Culture of Human Hepatic Endothelial Cells (HECs). HECs were isolated from 150 g of tissue as previously described.[14] Briefly, liver tissue was digested enzymatically with collagenase type 1A (Sigma), filtered, and further purified via density gradient centrifugation over 33%/77% Percoll (Amersham Biosciences). HECs were extracted from the mixed nonparenchymal population initially via negative magnetic selection with HEA-125 (50 μg/mL; Progen Biotechnik) to deplete biliary epithelial cells, and this was followed by positive selection with an anti-CD31 antibody conjugated to Dynabeads (10 μg/mL; Invitrogen, United Kingdom). $CD31^+$ endothelial cells were maintained after isolation in rat tail collagen–coated flasks (Sigma) in complete endothelial media (Gibco, Invitrogen, United Kingdom) supplemented with 10% heat-inactivated human AB serum (Invitrogen, United Kingdom) and with 10 ng/mL hepatocyte growth factor and 10 ng/mL vascular endothelial growth factor (both from PeproTech). HECs were grown until confluency and were used within five passages. The majority of cells isolated by this method expressed markers of sinusoidal endothelium, such as liver/lymph node–specific intercellular adhesion molecule 3–grabbing non-integrin and lymphatic vessel endothelial receptor 1.[21]

In order to determine whether HECs have characteristics consistent with vessels seen in the inflamed liver, we studied the expression of endothelial adhesion molecules with a cell-based enzyme-linked immunosorbent assay in HECs from normal (n = 3) and diseased livers (n = 3) according to the standard methodology.[14] The protocol and antibodies are listed in the Supporting Information Materials and Methods and Supporting Information Table 1. The expression of cytokeratin 19 (biliary epithelial cells), cytokeratin 18 (hepatocytes), CD68 (macrophages), and CD11c (dendritic cells) markers was used along with CD31

(endothelial cell marker) to confirm the purity of HEC cultures by flow cytometry. The antibodies are presented in the Supporting Information Materials and Methods and Supporting Information Table 2.

Isolation of Peripheral Blood Lymphocytes (PBLs). Peripheral venous blood from PSC patients with IBD was collected into ethylene diamine tetraacetic acid tubes, and lymphocytes were isolated by density gradient centrifugation over Lymphoprep (Sigma) according to the established methodology.[22]

Cell Lines and Culture Conditions

JY cells (a B-lymphoblastoid cell line expressing $\alpha 4\beta 7$) were grown in Roswell Park Memorial Institute 1640 medium (Invitrogen) containing l-glutamine and 10% fetal bovine serum (FBS; Invitrogen).

VAP-1–Dependent MAdCAM-1 Expression

Adenoviral Infection of Human HECs With VAP-1 Constructs. Adenoviral constructs encoding wild-type (WT) human vascular adhesion protein 1 (hVAP-1) and enzymatically inactive hVAP-1 [Tyr(Y)471Phe(F)] have been previously described.[23] Before their use, the enzymatic activity of VAP-1 transfectants was confirmed with the Amplex UltraRed method, which is described in the Supporting Information Materials and Methods. HECs were cultured until confluency, washed in phosphate-buffered saline to ensure the complete removal of human serum, and infected with the constructs at an optimal multiplicity of infection of 600 for 4 hours in endothelial basal medium 2 (Clonetics, Lonza) supplemented with 10% FBS. Transfected cells were then incubated with TNF-α (20 ng/mL; Peprotech) alone or in combination with MA (50 μM; Sigma-Aldrich) for 2 hours.

HEC Stimulation With End Products Released From MA Deamination by VAP-1/SSAO. Formaldehyde (HCHO), ammonia (NH_3), and hydrogen peroxide (H_2O_2) are produced during the VAP-1–catalyzed deamination of MA. In order to determine whether these end products had a role in the induction of MAdCAM-1, untransfected HECs were exposed to 1 or 10 μM H_2O_2 (BDH Prolabo), NH_3 (Merck; 8 M), or HCHO (J.T. Baker; 13.44 M) for 4 hours. In certain experiments, HECs were subjected to repeated dosing with H_2O_2 (8 times at 10 μM with 30-minute intervals) or to a combination of all three compounds, and their effect on MAdCAM-1 messenger RNA (mRNA) expression was analyzed. Viability assays confirmed that these treatments did not significantly alter endothelial viability after 4 hours of treatment.

Humanized VAP-1 Mice: VAP-1–Dependent Signaling In Vivo. WT mice and VAP-1–deficient mice (C57BL/6) expressing enzymatically active or inactive hVAP-1 on the endothelial cells under the control of the mouse tie 1 promoter have been described,[24] and they were used to study the role of VAP-1 in MAdCAM-1 induction *in vivo*. All mice were handled in accordance with the institutional animal care policy of the University of Turku.

MA [0.4% (wt/vol)] was administered in the drinking water of the animals (freshly made every day) for 14 days. After the mice were sacrificed, tissue samples from PPs and MLNs were excised and used for protein and RNA analysis.

Precision-Cut Liver Slice Organ Culture

In order to study MAdCAM-1 induction in the intact human liver, we used a Krumdieck tissue slicer (TCS Biologicals) to cut aseptic, 250-μm-thick slices of live liver tissue, which could be studied for up to 48 hours *ex vivo*. The liver tissue was incubated in Williams' E media (Sigma) supplemented with 2% FBS, 0.1μM dexamethasone (Sigma), and 0.5μM insulin (Novo-Nordisk). Tissues were stimulated with MA (50 μM) and enzymatically active recombinant vascular adhesion protein 1 (rVAP-1) produced in Chinese hamster ovary cells (500 ng/mL; Biotie Therapies, Turku, Finland) before MAdCAM-1 protein and RNA analysis. The viability of the excised tissue slices was tested with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) before and after the stimulation period (details are provided in the Supporting Information Materials and Methods).

RNA Analysis

Total RNA was extracted with the RNeasy mini Kit (Qiagen, United Kingdom) and analyzed as described in the Supporting Information Materials and Methods.

Protein Analysis

MAdCAM-1 protein expression was determined by western blotting and immunoprecipitation techniques. The protocols and antibodies are described in the Supporting Information Materials and Methods.

Immunohistochemical Analysis

Multicolor fluorescence confocal microscopy was used to localize the expression of MAdCAM-1 in HECs. MAdCAM-1 expression in human liver tissue was investigated in formalin-fixed, sucrose-embedded tissues with NovaRED immunostaining. The presence of murine MAdCAM-1 in PPs and MLNs was examined by immunofluorescence. The protocols and

antibodies are described in the Supporting Information Materials and Methods and Supporting Information Table 3.

Static Adhesion Assays

Formalin-fixed, sucrose-embedded sections (10 μm thick) were incubated with JY cells and PBLs from PSC patients (n = 3) for 30 minutes at room temperature. In certain experiments, tissue sections were incubated with an anti–MAdCAM-1 antibody (P1; 1 μg/mL; Pfizer), and JY cells and PBLs were blocked with anti-$\alpha4\beta7$ [actin 1 (ACT-1); 1 μg/mL; a gift from M. Briskin, Millenium, United States] for 30 minutes before the static assays. An isotype-matched control (IMC) antibody (immunoglobulin G1; 1 μg/mL; Dako) was used as a negative control. After several washes to remove unbound antibodies, sections were incubated with 10^5 JY or PSC PBLs/100 μL and resuspended in Roswell Park Memorial Institute 1640 medium plus 0.1% bovine serum albumin. Cells were allowed to bind under static conditions at room temperature for 30 minutes before they were washed, fixed in acetone, and counterstained with Mayer's hematoxylin (VWR International, Ltd.). Slides were analyzed via the manual counting of adherent lymphocytes in 40 representative high-power fields (with a 40× objective).

Flow-Based Adhesion Assays

The function of the MAdCAM-1 protein *in vitro* was studied with flow-based adhesion assays.[17] Briefly, confluent monolayers of HECs were cultured in microcapillaries and stimulated for 2 hours with TNF-α and MA before the perfusion of $\alpha4\beta7^+$ JY cells at a wall shear stress of 0.05 Pa. Adherent cells were visualized by phase contrast microscopy (with a 10× objective) and classified as rolling, static, or migrated cells. The total adhesion was calculated as cells per square millimeter normalized to the number of perfused lymphocytes. In function-blocking experiments, HECs were pretreated with a humanized anti-human P1 antibody (5 μg/mL), or JY cells were incubated with anti-$\alpha4\beta7$ (ACT-1; 1 μg/mL) for 30 minutes at 37°C. An IMC antibody (immunoglobulin G1; 1 μg/mL; Dako) was used as a negative control.

Statistical Analyses

Data were analyzed with the Student *t* test for comparisons of numerical variables between two groups and with one-way analysis of variance analysis followed by a Bonferroni post test for comparisons between more than two groups. Statistical analyses were performed with GraphPad Prism software. $P < 0.05$ was considered statistically significant.

Results

Purity and Phenotypic Characterization of HECs

We analyzed the purity of our HEC primary cultures and confirmed that more than 99% of HECs were $CD31^+$, with very few contaminating nonendothelial cells (Supporting Information Fig. 1A). As reported previously, HECs lack P-selectin but express minimal levels of E-selectin, low levels of vascular cell adhesion molecule 1 (VCAM-1), and high constitutive levels of intercellular cell adhesion molecule 1 (ICAM-1) and CD31, which are all increased upon inflammation.[25] We confirmed that under basal conditions, HECs isolated from nondiseased livers (two resections and one normal donor) and diseased livers [one with PSC, one with primary biliary cirrhosis (PBC), and one with alcoholic liver disease (ALD)] adopted a nonactivated phenotype expressing similarly high levels of ICAM-1 and CD31, low levels of VCAM-1 and E-selectin, and no P-selectin (Supporting Information Fig. 1B). Thus, in this study, we grouped together the data from all HECs used.

Treatment of HECs With the VAP-1/SSAO Substrate MA and TNF-α Induces MAdCAM-1 mRNA Expression, Protein Redistribution Onto the Cell Surface, and Increased Secretion of Soluble Mucosal Addressin Cell Adhesion Molecule 1 (sMAdCAM-1)

Using quantitative polymerase chain reaction (PCR), we detected significantly higher MAdCAM-1 mRNA levels in HECs stimulated with TNF-α alone and in combination with MA versus unstimulated HECs (Fig. 1A). Total cell MAdCAM-1 protein levels were unaffected by stimulation, and no detectable increase in cytoplasmic MAdCAM-1 was observed either; this was confirmed by western blotting and flow cytometry (data not shown). However, using live cell staining and confocal microscopy, we observed that the MAdCAM-1 protein was redistributed onto the surface of HECs stimulated with TNF-α and MA (Fig. 1B). In addition, we found that MAdCAM-1 was released in a soluble form (sMAdCAM-1) in the supernatant of TNF-α–treated and MA-treated HECs in comparison with media alone (Fig. 1C). Therefore, we have shown that MA and TNF-α up-regulate MAdCAM-1 mRNA expression in HECs, induce protein redistribution onto the cell surface, and promote increased secretion of sMAdCAM-1.

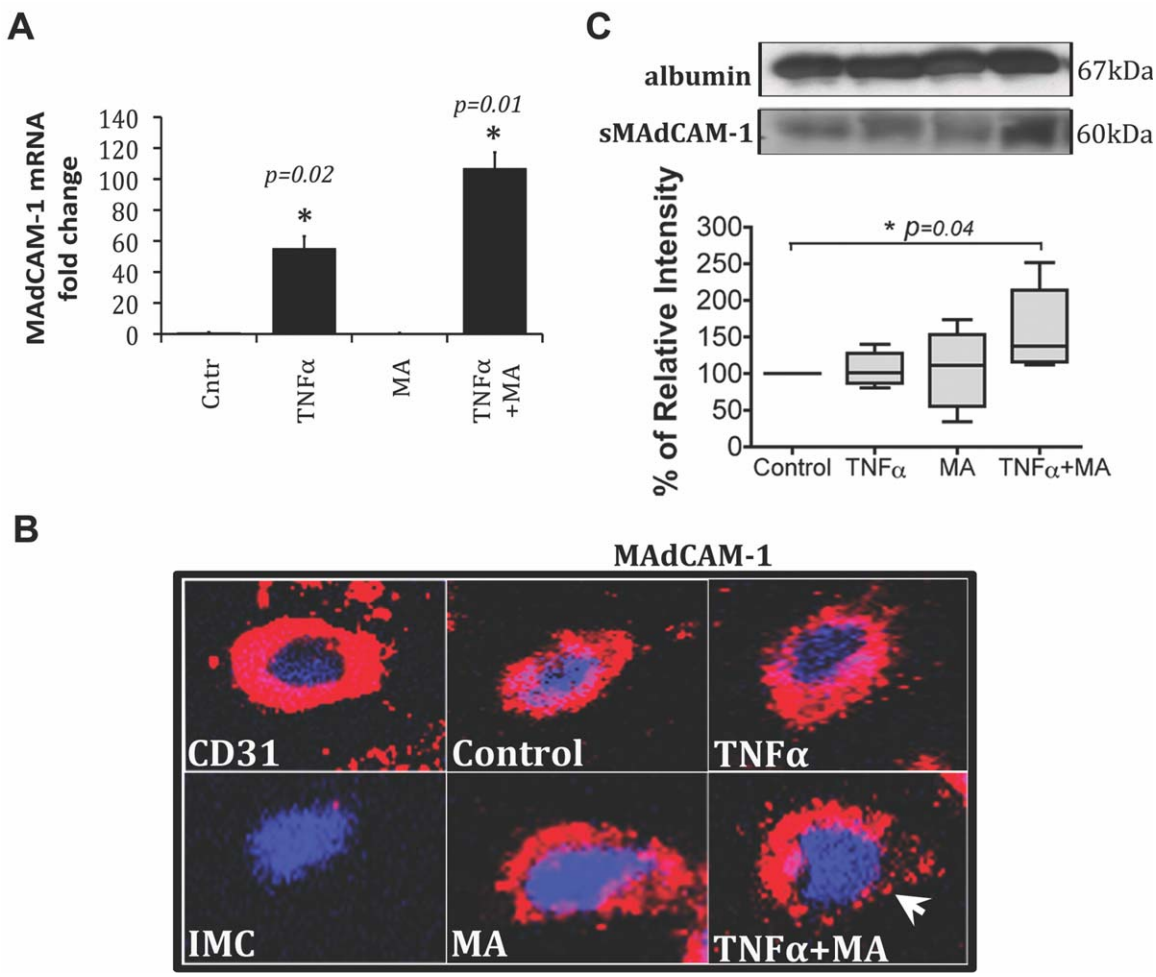

Fig. 1. The physiological VAP-1 substrate MA potentiates TNF-α–induced expression of MAdCAM-1 in HECs. Endothelial cells were stimulated with TNF-α (20 ng/mL), MA (50 μM), or a combination of the two for 2 hours. (A) mRNA analysis by quantitative PCR. Data represent MAdCAM-1 mRNA fold changes in stimulated HECs versus a control (no stimulation). Means and standard deviations are shown (n = 7 HECs). *$P < 0.05$ versus the control by the Student *t* test. (B) Confocal microscopy images of unstimulated live HECs (control) and HECs stimulated with TNF-α and MA (either alone or in combination). The localization of the MAdCAM-1 protein on the cell surface is shown in red. CD31 (red) was used as a positive control, and an IMC antibody was used as a negative control. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). The original magnification was ×40. The white arrow indicates MAdCAM-1 redistribution onto the cell surface after TNF-α and MA stimulation. (C) MAdCAM-1 was immunoprecipitated from the cell culture supernatants of the control and 2-hour–stimulated cells. sMAdCAM-1 was captured with the polyclonal antibody (H-116), and a 60-kDa molecular weight band was detected with the monoclonal antibody (CA102.2C1). Densitometric analysis was performed for six different HEC isolates. Data represent the mean percentages and standard errors of the mean of the relative intensity normalized to the serum albumin levels and compared to the control (set to 100%). *$P = 0.04$ versus the sMAdCAM-1 levels in the control by the Student *t* test.

MAdCAM-1 Expressed by HECs Is Functionally Active

To study the function of HEC-expressed MAdCAM-1, we used flow-based adhesion assays with JY cells, which express high levels of the MAdCAM-1 receptor α4β7 on the cell surface (Fig. 2A). JY cells were perfused over HEC monolayers at 0.05 Pa, and adhesion was recorded. Under basal conditions, no adhesion was detected; however, stimulation of HECs with TNF-α and MA significantly increased the total number of adherent cells, and this was reduced by an antibody blockade of MAdCAM-1 (P1) or α4β7 (ACT-1; Fig. 2B). The IMC antibody that was used showed no inhibitory effect [109 ± 21 adherent cells/mm^2/10^6 perfused cells (standard error of the mean) in HECs treated with TNF-α and MA and 116 ± 41 adherent cells/mm^2/10^6 perfused cells in TNF-α and MA and isotype control stimulated HEC]. Altogether, our data show that TNF-α and MA induce the redistribution of the MAdCAM-1 protein onto the cell surface and render it functionally active to support the binding of α4β7$^+$ JY cells.

VAP-1/SSAO Enzyme Activity Induces Endothelial Expression of MAdCAM-1 In Vitro

To validate the role of VAP-1/SSAO in MAdCAM-1 induction, we used adenoviral constructs encoding

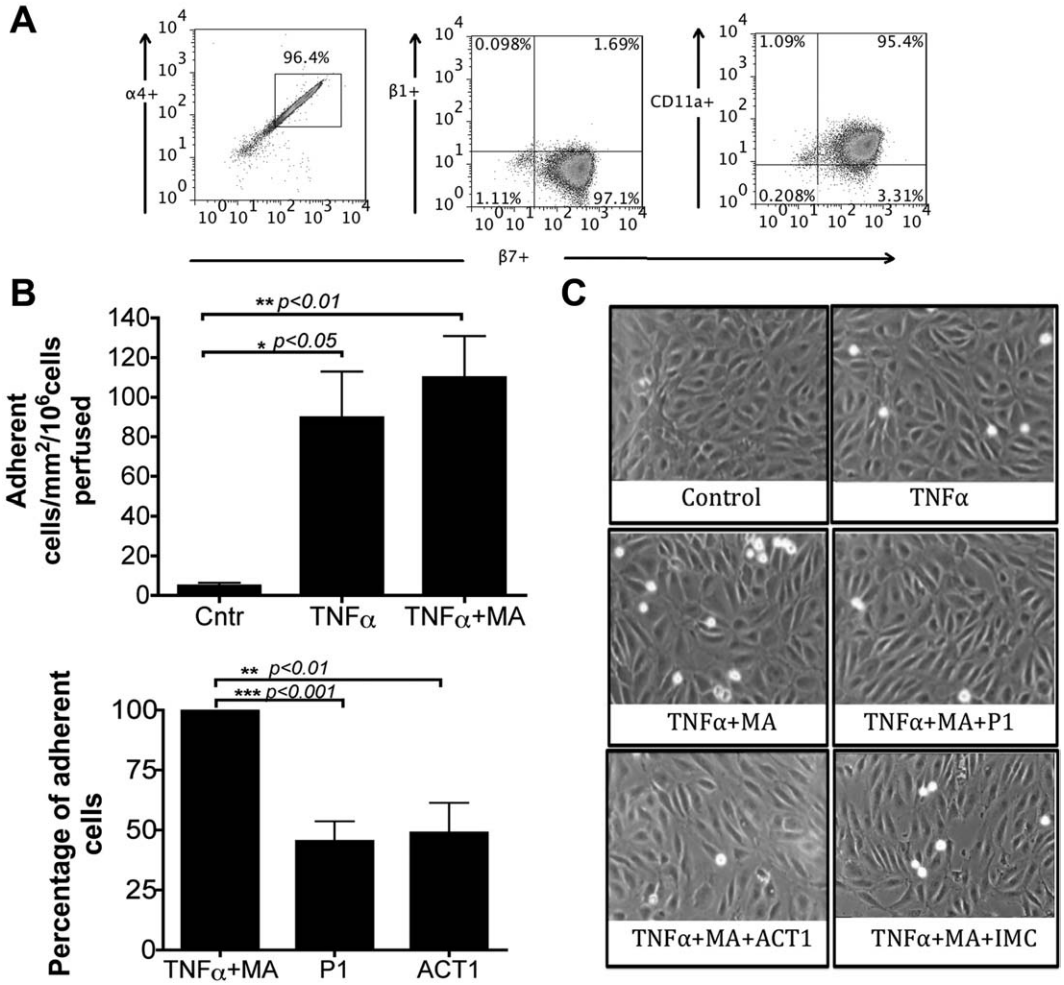

Fig. 2. MAdCAM-1 expressed by HECs supports lymphocyte adhesion in a flow-based adhesion assay. (A) Representative flow cytometry dot plots show the coexpression of $\alpha 4$, $\beta 1$, and CD11a with $\beta 7$ integrin on JY cells. (B) HECs were treated with TNF-α (20 ng/mL) alone or in combination with MA (50 μM) for 2 hours, and JY cells were perfused over the monolayer at a shear stress of 0.05 Pa. Adhesion was blocked by function-blocking antibodies directed against MAdCAM-1 (P1; 5 μg/mL; HECs) or $\alpha 4\beta 7$ (ACT-1; 1 μg/mL; JY cells). Data represent the mean adhesion values and standard errors of the mean from seven different HECs. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by the Student *t* test. (C) Representative images captured from experimental videos show adherent cells in the absence and presence of function-blocking antibodies (P1 and ACT-1) or an IMC.

enzymatically active and inactive hVAP-1. The enzyme activities of the constructs were confirmed before use (Supporting Information Fig. 2). More than 95% of HECs transfected with the adenoviral constructs expressed hVAP-1 on their surface (Fig. 3A), with similar median channel fluorescence values for the two constructs (197 $\pm$ 40 for hVAP-1 and 216 $\pm$ 40 for hVAP-1_Y471F, n = 7 HECs). We then exposed transfected HECs to MA and TNF-α and observed increased MAdCAM-1 protein levels in HECs transfected with enzymatically active hVAP-1 (Fig. 3B1). Under control conditions, the presence of WT hVAP-1 caused a significant increase in comparison with HECs transfected with the mutant hVAP-1, probably as a result of endogenous ligands. When HECs were stimulated with TNF-α and MA in the presence of WT hVAP-1, there was a significant increase in MAdCAM-1 expression in comparison with HECs transfected with mutant hVAP-1 (Fig. 3B2).

To further confirm the role of VAP-1/SSAO in MAdCAM-1 induction, we studied the effects of the end products released by MA deamination by VAP-1. Untransfected HECs were stimulated with the MA metabolites H_2O_2, NH_3, and HCHO for 4 hours, at which time more than 98% of the cells were viable (data not shown). When H_2O_2 was administered repeatedly every 30 minutes at 10 μM with the other end products, there was a significant 10-fold increase in MAdCAM-1 expression (Fig. 3C). Therefore, our data show that the enzymatic activity of VAP-1 can up-regulate MAdCAM-1 expression in HECs.

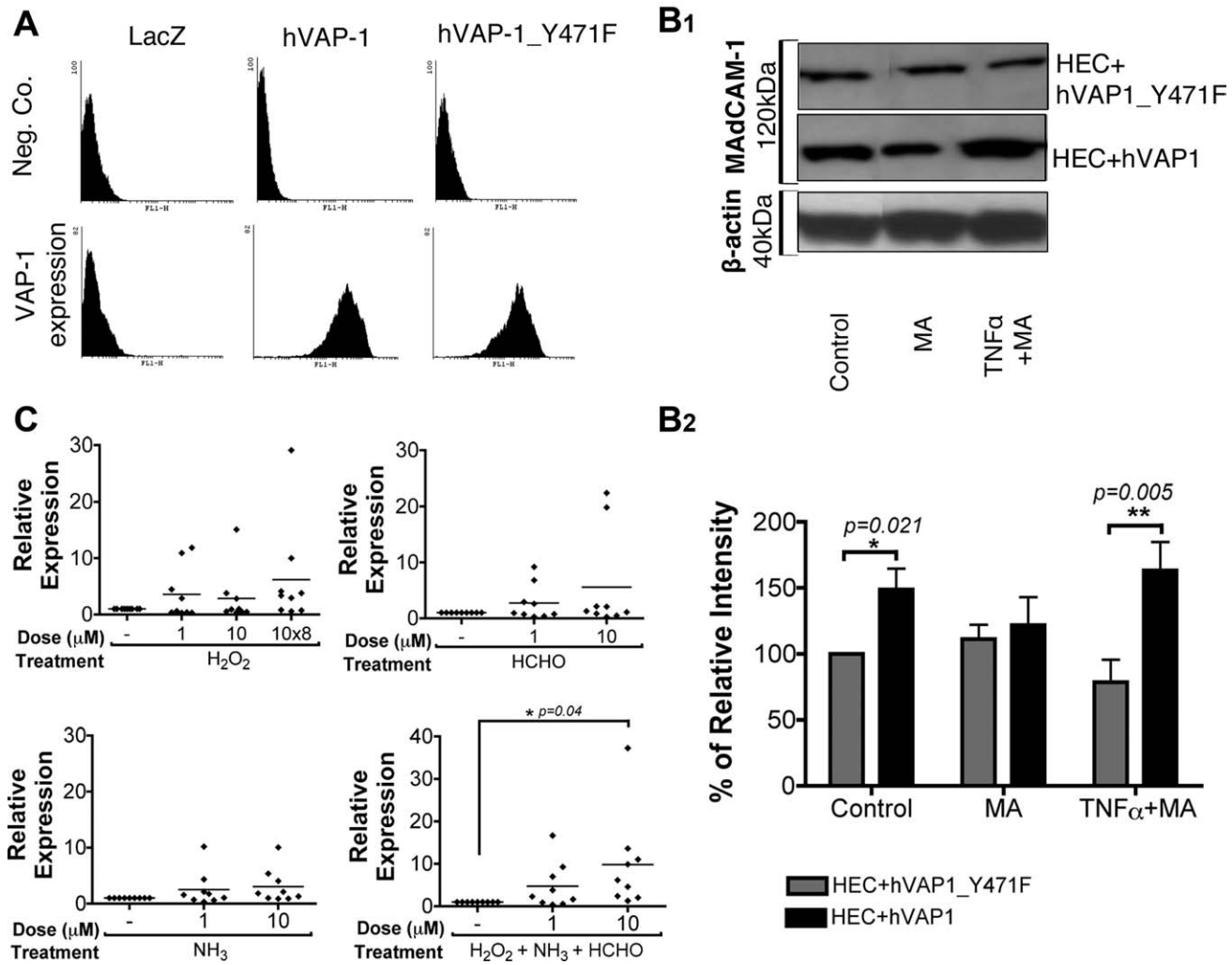

Fig. 3. VAP-1/SSAO induces MAdCAM-1 expression *in vitro*. (A) HECs were transfected with adenoviral constructs expressing WT hVAP-1 or an enzymatically inactive mutant of hVAP-1 (hVAP-1_Y471F). VAP-1 positivity on transfected HECs was confirmed by flow cytometry with anti–VAP-1 or negative control mouse anti-human antibodies. Data are representative of seven different HECs. (B) After adenoviral transfection, HECs were stimulated with TNF-α (20 ng/mL) or MA (50 μM) for 2 hours before lysis and western blotting: (1) representative blots of the 120-kDa MAdCAM-1 dimeric protein and 40-kDa β-actin and (2) densitometric analysis of seven different HECs. Data represent the mean percentages and standard errors of the mean of the MAdCAM-1 protein expression normalized to endogenous β-actin levels and are shown with respect to the expression in unstimulated (control) HECs transfected with mutant hVAP-1 (HEC+hVAP-1_Y471F; set to 100%). Statistical analysis was performed with the Student *t* test. $*P = 0.021$ versus control HEC+hVAP-1_Y471F and $**P = 0.005$ versus HEC+hVAP-1_Y471F stimulated with TNF-α and MA. (C) End products of VAP-1 enzyme activity induced MAdCAM-1 expression by HECs. Untransfected HECs were stimulated with single doses of H_2O_2, HCHO, and NH_3 at 1 or 10 μM for 4 hours. As indicated, HECs were treated repeatedly with 10 μM H_2O_2 (8 times every 30 minutes; 10 $\times$ 8) or with a combination of all three end products (each at 1 or 10 μM single doses and H_2O_2 added repeatedly). RNA was extracted from cells, and MAdCAM-1 mRNA expression was measured with quantitative PCR. Data represent the relative expression of treated HECs versus untreated HECs (the mean is shown as a horizontal line; the data were taken from nine different HECs). $*P = 0.04$ versus the control by one-way analysis of variance.

VAP-1/SSAO Induces Human Hepatic MAdCAM-1 Expression Ex Vivo

To validate the *in vitro* effects of VAP-1/SSAO signaling, we used a liver organ culture system in which viable, precision-cut human liver slices were stimulated with rVAP-1 and MA. Initially, we studied the expression of MAdCAM-1 in normal liver tissues and diseased liver tissues [PBC, ALD, PSC, and autoimmune hepatitis (AIH)] and found higher MAdCAM-1 expression levels in chronic liver diseases (Fig. 4A); this agreed with previous reports.[10] We then stimulated normal liver tissue slices with rVAP-1 and its substrate MA to see whether increased enzyme activity would induce MAdCAM-1 expression. Time course studies detected increased MAdCAM-1 protein expression, which peaked at 4 hours; this was followed by a decline until 8 hours of treatment (Fig. 4B). rVAP-1 and MA caused a significant increase in MAdCAM-1 mRNA levels in normal liver tissue (n = 4; Fig. 4C) and increased MAdCAM-1 protein expression in

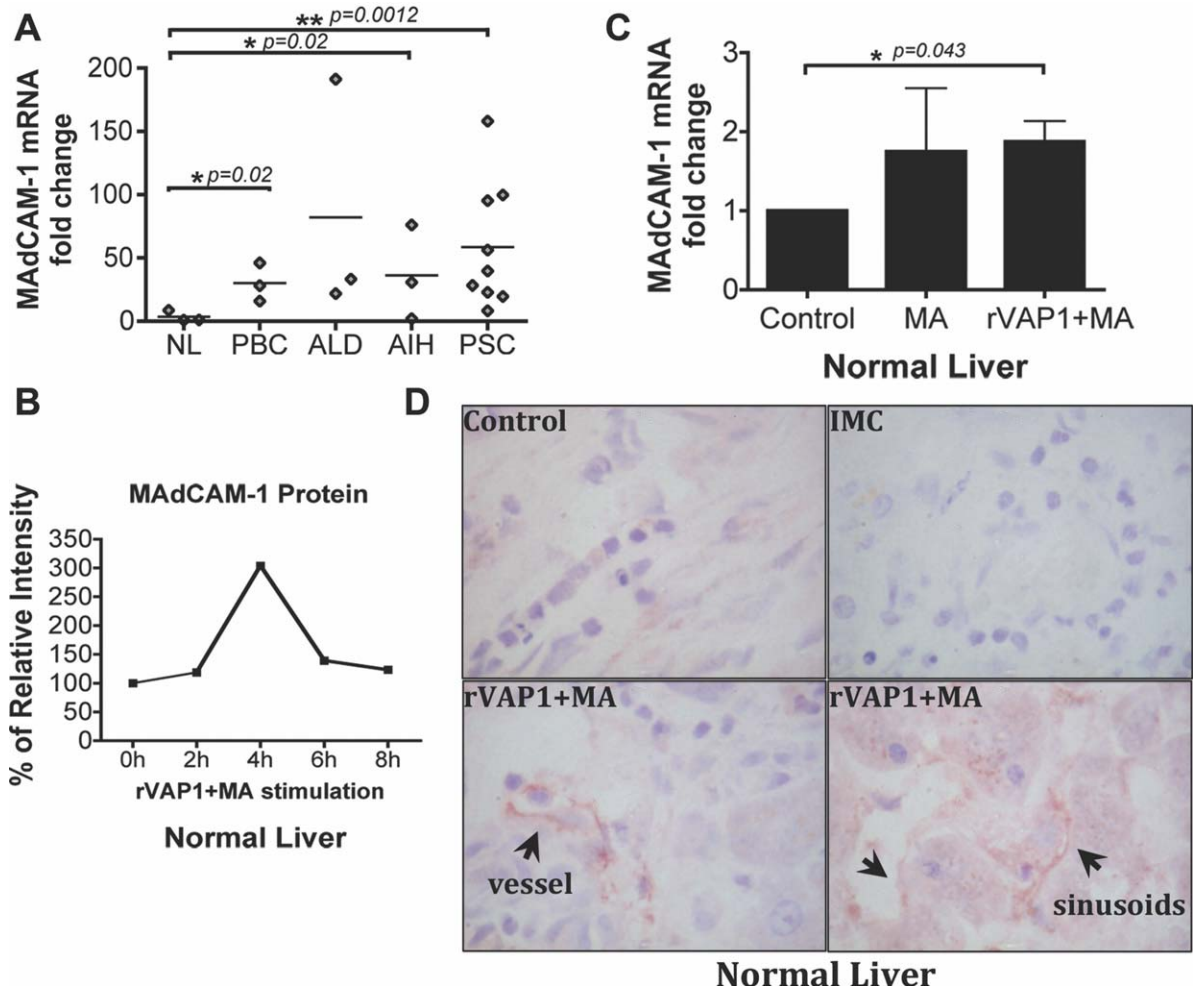

Fig. 4. MAdCAM-1 is expressed in the diseased human liver, and expression is increased in the normal liver (NL) by VAP-1/SSAO activity *ex vivo*. (A) MAdCAM-1 mRNA expression in NL (n = 3), PBC (n = 3), ALD (n = 3), AIH (n = 3), and PSC (n = 9) tissues: MAdCAM-1 mRNA levels (means and standard deviations) in diseased livers with respect to NL tissues (the mean is shown as a horizontal line). $*P < 0.05$ and $**P < 0.01$ versus expression in NL by the unpaired Student *t* test. (B) NL tissue slices were stimulated with rVAP-1 (500 ng/mL) and MA (50 μM) for 0 to 8 hours, and the MAdCAM-1 protein was analyzed by western blotting. Data represent the percentage of MAdCAM-1 protein expression normalized to the endogenous β-actin levels. (C) Precision-cut NL tissue slices (250 μm) from four different livers were stimulated with MA and rVAP-1 for 4 hours, and MAdCAM-1 mRNA expression was analyzed by quantitative PCR. MAdCAM-1 mRNA fold changes in treated samples versus control samples are shown. $*P < 0.05$ versus the control by the Student *t* test. (D) MAdCAM-1 protein expression in vessels and sinusoids (black arrows) on a representative untreated NL (control) or an NL stimulated with MA and rVAP-1. The original magnification was $\times$600.

vessels (Fig. 4D). An MTT assay also revealed >91% viability after 4 hours of stimulation (data not shown). To show that the induced MAdCAM-1 was functional, we used static adhesion assays, and we demonstrated increased $\alpha4\beta7^+$ JY cell binding to hepatic vessels in tissues stimulated with rVAP-1 and MA (Fig. 5A); this was reduced by the pretreatment of tissues with an anti–MAdCAM-1 antibody (P1) or lymphocytes with $\alpha4\beta7$ (Fig. 5C,E). We then confirmed the findings with PBLs from PSC patients with IBD; these cells adhered efficiently to tissues stimulated with rVAP-1 and MA (Fig. 5B), and again, this was blocked by anti–MAdCAM-1 (P1) and anti-$\alpha4\beta7$ (ACT-1; Fig. 5D). The IMC antibody did not cause any reduction in adhesion (Fig. 5C,D). Thus, these data confirm that VAP-1/SSAO can induce the expression of functionally active human hepatic MAdCAM-1 *ex vivo*, which is able to regulate lymphocyte recruitment to the liver.

VAP-1/SSAO Induces MAdCAM-1 Expression In Vivo

To investigate the role of VAP-1/SSAO–dependent MA deamination in MAdCAM-1 expression *in vivo*, we used WT mice and VAP-1–deficient mice expressing hVAP-1 in an enzymatically active or inactive form as a transgene in endothelial cells. The presence of hVAP-1 in the livers of transgenic animals was confirmed by immunofluorescent staining (Fig. 6A). To test whether MA could alter MAdCAM-1 expression *in vivo*, it was given

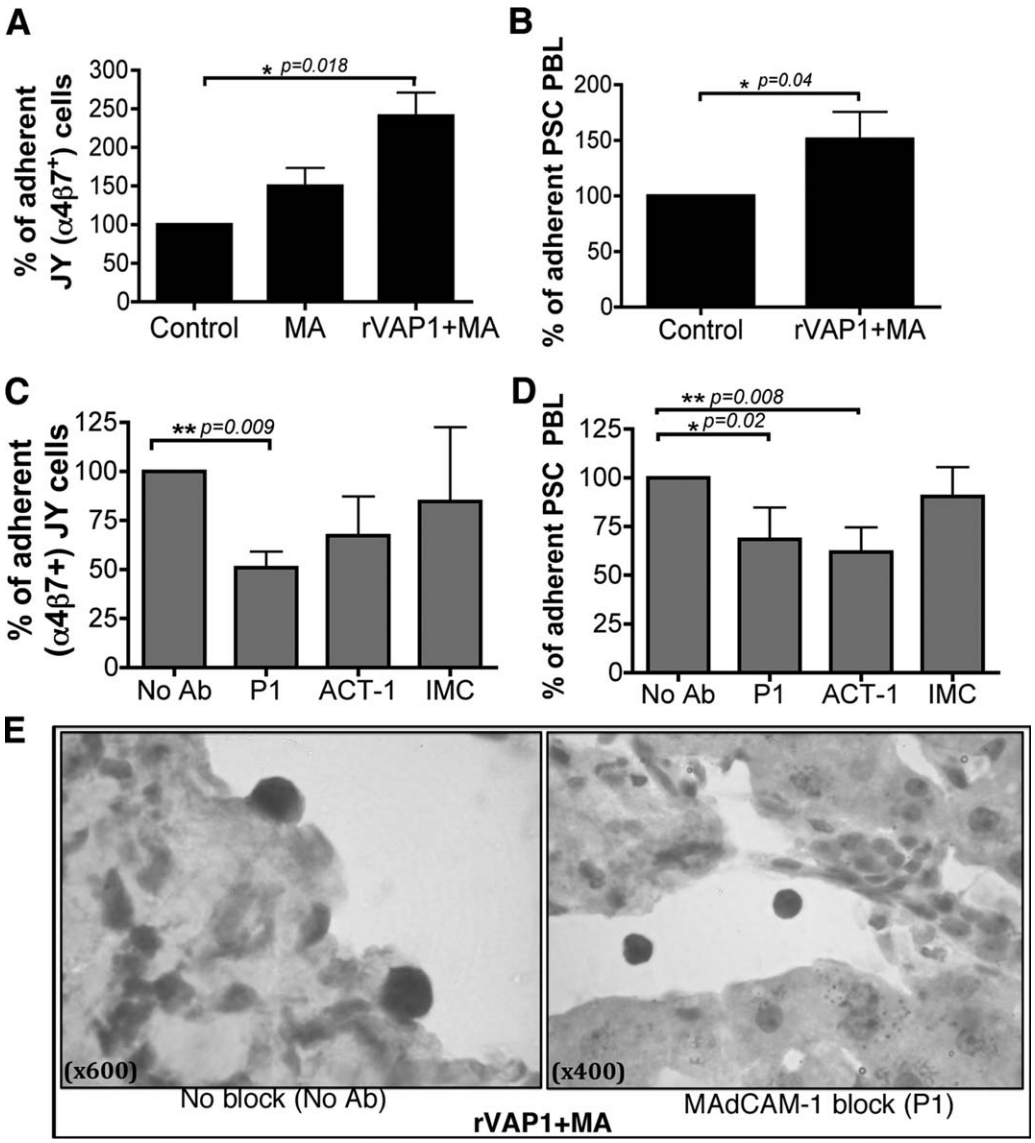

Fig. 5. MAdCAM-1 induced in the normal liver supports the adhesion of JY cells and PBLs from PSC patients. Static adhesion assays show the adhesion of (A) $\alpha 4\beta 7^+$ JY cells and (B) PBLs from PSC patients to normal liver sections cut from precision-cut liver slices after 4 hours of stimulation with rVAP-1 and MA. Data represent the mean adhesion values and standard errors of the mean from four normal livers and three PSC PBLs. (C,D) Binding was inhibited when MAdCAM-1 (P1; 1 μg/mL) and $\alpha 4\beta 7$ (ACT-1; 1 μg/mL) were blocked. Data show the percentage of adherent cells versus control samples; when blocking was used, data show the percentage of adherent cells versus adhesion in samples for which no antibody was used (No Ab). An IMC was used at 1 μg/mL. $^*P < 0.01$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by the Student *t* test. (E) Representative immunostaining pictures show adherent lymphocytes in rVAP-1–stimulated and MA-stimulated normal liver tissue and nonadhering lymphocytes in tissues in which MAdCAM-1 was blocked (P1). Images were acquired at the original magnifications of $\times 600$ and $\times 400$, respectively.

to the animals through their drinking water for 14 days. We were unable to detect MAdCAM-1 mRNA or protein in the murine liver before or after stimulation in all animal models by mRNA analysis, western blotting, and immunofluorescence (data not shown). However, we detected significant 10- and 16-fold increases in MAdCAM-1 mRNA levels and increased MAdCAM-1 protein levels in PPs and MLNs of transgenic animals expressing enzymatically active hVAP-1 after MA administration (Fig. 6A,B). The importance of VAP-1/SSAO in this induction was confirmed by studies showing reduced MAdCAM-1 mRNA induction in mice expressing the enzymatically inactive form of hVAP-1 (Fig. 6B). Therefore, these data demonstrate the ability of VAP-1 enzyme activity to induce MAdCAM-1 expression in gut mucosal vessels *in vivo*.

Discussion

The ability of aberrantly expressed hepatic MAdCAM-1 to recruit mucosal T cells to the liver in patients with PSC[9,10] led us to further investigate

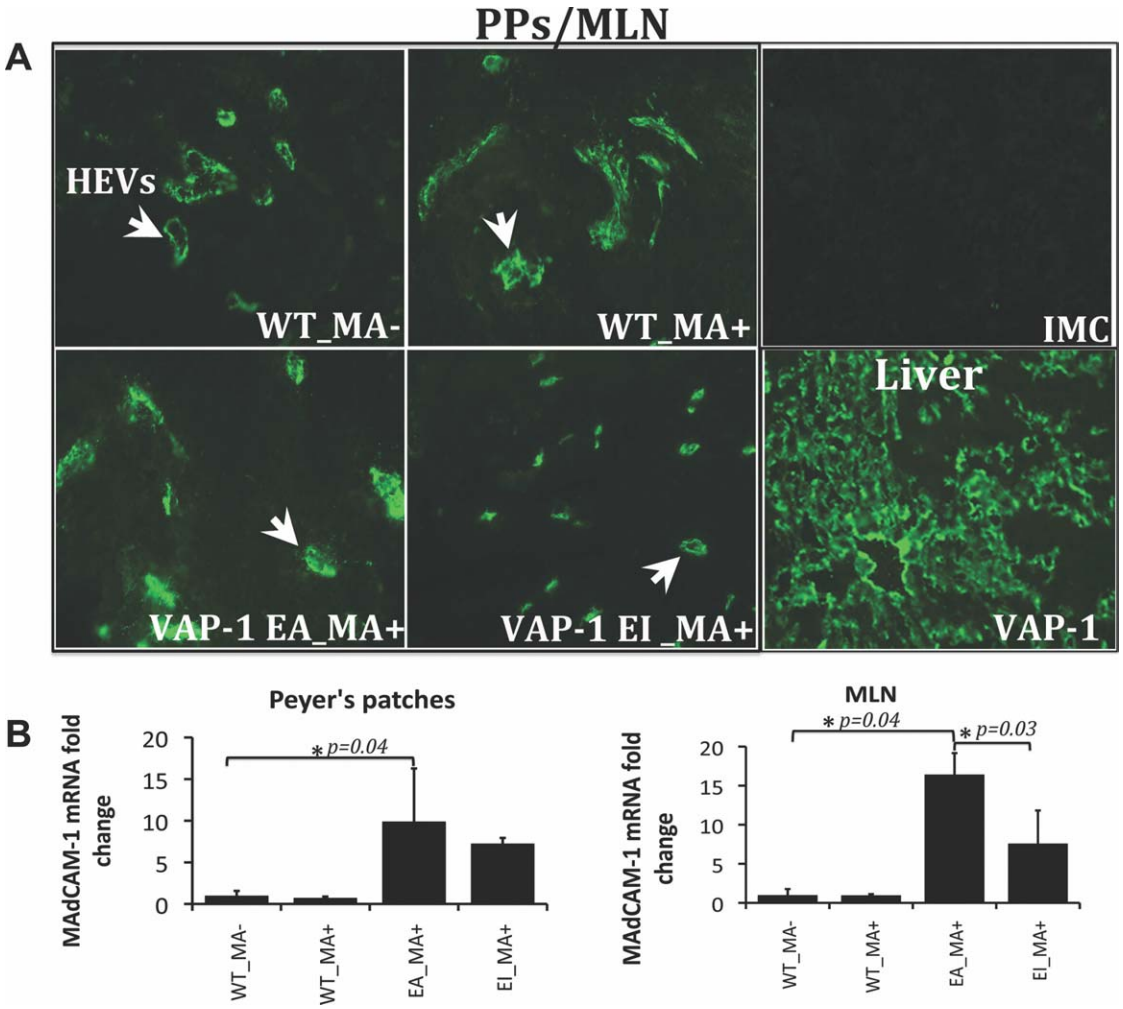

Fig. 6. VAP-1/SSAO induces MAdCAM-1 expression *in vivo*. WT mice and VAP-1–deficient mice expressing enzymatically active (VAP-1_EA) and inactive (VAP-1_EI) hVAP-1 were supplied with 0.4% (wt/vol) MA (MA^+) in their drinking water for 14 days. WT mice without MA treatment were used as controls (WT_MA^-). (A) Immunofluorescent staining of MAdCAM-1 in HEVs (white arrows) of PPs and MLNs and *in vivo* expression of hVAP-1 in the livers of transgenic mice. An IMC antibody was used as a negative control. (B) MAdCAM-1 mRNA analysis with real-time PCR. Data represent MAdCAM-1 mRNA fold changes (means and standard deviations) in PPs and MLNs in mice ($n = 3$ in each group) receiving MA versus WT_MA^- mice ($n = 3$). $^*P < 0.04$ in both PPs and MLNs of EA_MA^+ mice versus WT_MA^- mice by the Student *t* test and $^*P = 0.03$ in MLNs of EA_MA^+ mice versus EI_MA^+ mice by the Student *t* test.

factors involved in hepatic MAdCAM-1 induction. In this study, we provide evidence that VAP-1/SSAO–dependent oxidation of MA increases MAdCAM-1 expression in HECs *in vitro* and *ex vivo* and in mucosal vessels *in vivo*. These findings implicate VAP-1/SSAO activity in inducing and maintaining MAdCAM-1 expression in the gut and the liver.

Although provision of the VAP-1 substrate MA or TNF-α led to induction of MAdCAM-1, the combination of the stimuli had an additive effect. The role of TNF-α in MAdCAM-1 induction has been reported previously in both *in vitro* and *in vivo* systems.[18-20] However, it is unlikely that TNF-α alone is sufficient to induce hepatic MAdCAM-1 *in vivo* because hepatic MAdCAM-1 expression is limited, with the strongest and most consistent expression seen in patients with PSC or AIH complicating IBD.[10] This led us to look for other factors that may have a particular role in the liver. VAP-1 is constitutively expressed in the human liver, and we have previously reported that the enzymatic activity of VAP-1 generates products (including H_2O_2) that can activate NF-κB–dependent adhesion molecule expression.[17] This led us to hypothesize that the VAP-1/SSAO enzymatic activity could also promote MAdCAM-1 expression. We now confirm that this is the case, and we further demonstrate that the natural VAP-1/SSAO substrate MA, which is present in food, wine, and cigarette smoke, is able to increase MAdCAM-1 expression *in vitro*, *in vivo*, and *ex vivo*.

Human HECs exposed to TNF-α and MA showed increased MAdCAM-1 mRNA transcription, protein redistribution onto the cell surface, and increased

secretion of the sMAdCAM-1 protein. Using flow-based adhesion assays, we confirmed that MA/TNF-α–induced MAdCAM-1 on HECs was functionally active and able to support increased adhesion of α4β7-expressing JY cells. There was residual binding of JY cells after MAdCAM-1 or α4β7 blocking, which we believe was mediated by lymphocyte function-associated antigen 1/ICAM-1. We also found that TNF-α and MA stimulation induced the production of a soluble form of MAdCAM-1. Leung et al.[26] first reported sMAdCAM-1 in human serum, urine, and other biological fluids, but it is not known whether this soluble form is functional. Soluble forms of other adhesion molecules, including E-selectin and VAP-1, have the ability to enhance adhesion to endothelium.[27,28] Therefore, sMAdCAM-1 produced via the action of VAP-1/SSAO could also serve as an attractant increasing leukocyte adhesion.

In addition to functioning as an adhesion molecule, VAP-1 is also an enzyme, and this led us to investigate whether this enzyme activity is critical for MAdCAM-1 induction. We present several pieces of experimental data to support this: (1) the provision of MA and TNF-α to HECs overexpressing enzymatically active hVAP-1 increased MAdCAM-1 expression, whereas HECs expressing enzymatically inactive hVAP-1 did not respond, and (2) the treatment of HECs with the end products of VAP-1 deamination of MA (HCHO, NH_3, and H_2O_2) increased MAdCAM-1 expression 10-fold. Local H_2O_2 has been implicated in the regulation of adhesion molecule expression.[29-32] We have reported that the end products of SSAO deamination (including H_2O_2) induce expression of endothelial E- and P-selectins in vascular endothelium[32] and expression of ICAM-1, VCAM-1, and chemokine (C-X-C motif) ligand 8 in human hepatic sinusoidal endothelium through stimulation of the phosphoinositide 3-kinase, mitogen-activated protein kinase, and NF-κB pathways.[17] Thus, H_2O_2 released as a result of MA deamination by VAP-1 could operate through the NF-κB binding elements present in the human MAdCAM-1 promoter region[33] to induce MAdCAM-1 expression.

The studies using primary HECs were compelling, but we wanted to see if MA could induce functional MAdCAM-1 in intact liver tissue. To do this, we used a novel liver organ culture system in which we could culture viable human liver tissue slices for up to 48 hours *ex vivo*. The addition of MA to cultures of normal human liver tissue resulted in VAP-1/SSAO–dependent induction of MAdCAM-1 RNA and protein on hepatic endothelium. Furthermore, we were able to confirm that the induced MAdCAM-1 was functional because it supported the adhesion of PBLs from patients with PSC to vessels in the tissue slices via the α4β7 integrin, which is expressed by up to 40% of circulating T cells in patients with PSC.[34]

Finally, we wanted to confirm the ability of VAP-1/SSAO to induce MAdCAM-1 *in vivo*. To do this, we used mice but found that we were unable to detect or induce any MAdCAM-1 in the murine liver. This finding agreed with reports from Bonder et al.,[13] who failed to detect MAdCAM-1 in murine portal venules and sinusoids after concanavalin A administration. This is a clear difference between mice and humans and might explain why it has been difficult to develop a representative murine model of PSC. However, MAdCAM-1 is expressed in mucosal vessels in mice, in which it is increased by inflammation. We now report that MA feeding increased MAdCAM-1 expression in HEVs of PPs and MLNs, and we confirmed that this induction was dependent on the enzymatic activity of VAP-1/SSAO because overexpression of enzymatically active endothelial VAP-1 in transgenic animals led to a significant increase in MAdCAM-1, which was reduced in animals expressing enzymatically inactive hVAP-1. Interestingly, WT animals did not show consistent responses to MA, and this probably reflects the relatively low levels of VAP-1/SSAO present in the absence of inflammation. Surprisingly, increased levels of MAdCAM-1 were detected in transgenic animals expressing enzymatically inactive hVAP-1. Although these levels were not generally as high as those seen in mice overexpressing enzymatically intact hVAP-1, we suggest that VAP-1 might also induce MAdCAM-1 by acting as an adhesion molecule and recruiting lymphocytes that then secrete factors promoting MAdCAM-1 induction.

In conclusion, our data reveal that VAP-1/SSAO contributes to MAdCAM-1 induction in HECs *in vitro* and *ex vivo* in humans and in gut mucosal vessels *in vivo* in mice. On the basis of these findings and previous reports describing the induction of VAP-1 during gut inflammation,[16] we suggest that MA at increased levels due to enhanced absorption via an inflamed gut or cigarette smoke[15] acts as a substrate for VAP-1/SSAO and thus leads to MAdCAM-1 expression in the inflamed gut mucosa and hepatic endothelium. This could promote the uncontrolled recruitment of mucosal effector cells and result in tissue damage that is characteristic of both IBD and its hepatic complications. Thus, targeting VAP-1/SSAO therapeutically could not only reduce lymphocyte adhesion directly but could also down-regulate MAdCAM-1 expression and lead to the resolution of both liver and gut inflammation.

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